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THE MECHANISM OF PHAGOCYTOSIS *

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INTRODUCTION

It can hardly be denied that for some years we have been under the impression that the phagocytic activity of certain metazoan body cells is analogous to that exhibited in the selective feeding reactions of many protozoa. The theory of Metschnikoff is based apparently on numerous observations which give support to such a conception. The writings of his pupils, Bordet, Burnett, and others, show clearly that they too have been hampered by such views. The very in vitro technic by which we are to study the activity of the phagocytes in taking up bacteria is based on this idea, else why incubate the mixture? It may be possible that some of the recorded observations of previous workers have been correctly interpreted, but we feel that in the light of the following experiments it will be necessary for us to alter our conception of the mechanism of phagocytosis.

Instead of the prevalent conception, we suggest that foreign particles, as carbon, carmin granules, etc., are "taken up" by leukocytes because the latter, as amebae of the limax group in the trophozoit stage, have sticky surfaces. The cell in its movements crawls over or

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against such particles, which in turn adhere to the cell surface and then are carried into the interior by cytoplasmic streaming currents. That amebae of the limax group, while in the trophozoit stage, are provided with sticky surfaces was noted by Sellards,[1] while working with the Barber pipette, and independently by Kite,[2] who further observed that this ameba (Vahlkampfia sp.) was not sticky while in the motile, flagellated stage. Furthermore, Kite[3] has noted that polymorphonuclear leukocytes also show a similar glass-adhesiveness under in vitro conditions, and he believes that different cells show considerable differences in degree of adhesiveness.

The fact that leukocytes are only barely able to take up many kinds of bacteria under in vitro conditions without the aid of something present in the body fluids which acts upon the bacteria rather than upon the phagocytes is now well known through the work of Wright, Hektoen, and others, and we believe that our experiments show clearly that "opsonins" act by making the bacterial membrane or capsule more sticky, so that the bacteria have a greater tendency to adhere to the surface of leukocytes which comes into contact with them. Some of the classical experiments of in vitro phagocytosis are here reproduced by forcing leukocytes through a bacterial emulsion by centrifugal force, and by less violent agitation; in fact, we have been unable to make a mixture of "opsonized" bacteria and leukocytes without getting some "phagocytosis."

The amount of adhesion in a mixture of bacteria, leukocytes, and unheated serum must depend, it seems to us, on the relative stickiness of the bacteria and the leukocytes.

The stickiness of the leukocytes we used in our in vitro experiments varied widely. For example, in Experiment 17, where cocci and unheated serum were incubated separately from washed leukocytes suspended in unheated serum and then the two suspensions agitated together, there was a marked variation in the number of bacteria taken up by individual leukocytes. In the fifty leukocytes counted in the smear, three contained no cocci, ten, 16-20 cocci, and the rest varied between 3-14 cocci. Here, an eosinophil containing 19 cocci and a large mononuclear with 6 cocci were noted. These were leukocytes from normal peripheral blood. It seems probable that this variation will be found to be much more marked in exudates and may reach such a degree as to allow the adhesion of some bacteria without opsonization. In this connection, we will cite the counts on 100 cells in the osmic preparation in Experiment 9, where washed aleuronat frog's leukocytes and the potato bacillus were agitated together. This count showed sixty-six leukocytes with no bacilli; twenty-six, with 1-10 bacilli; three, with 15-16 bacilli; five, with 20-43 bacilli, and, as shown under the details of the experiment, certain clumps of leukocytes often showed a considerable degree of adhesion. The argument which might be invoked against the results of Experiment 17, that there was not enough opsonin present to treat all the cocci or that they were treated unequally, can hardly be used against Experiment 9 unless it can be shown that opsonin comes from the leukocytes. Similar observations by Tunnicliff on the phagocytosis of the bacillus fusiformis and by Davis on the phagocytosis of influenza-like bacilli by washed leukocytes is mentioned by Hektoen.[4] Numerous workers have called attention to the variation in phagocytic power shown by leukocytes collected from normal and diseased animals. This work has been exhaustively reviewed by Hektoen.[5] We will refer here only to the observations of Rosenow,[6] showing that pneumonic leukocytes are more active in their phagocytic power than leukocytes from normal blood. He also noted that such leukocytes "are more actively phagocytic for certain rare strains of pneumococci in the absence of serum."[7] With regard to the greater stickiness of leukocytes in exudates, Tunnicliff[8] showed that the leukocytes in aleuronat exudates were more active phagocytically than the leukocytes obtained from the blood of the same animals.

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[1] Philippine Jour. Sc., Series B, 1911, 23, p. 81.
[2] See Wherry: Arch. f. Protistenk., 1913, p. 77.
[3] Jour. Infect. Dis., 1914, 15, p. 319.

We believe that Experiments 8, 10, and 11, made with a minimum of agitation, are very significant, for here practically just as dense suspensions of bacteria were used as in the other experiments, and yet they yielded comparatively low counts. The two experiments on direct observation of the mechanism of phagocytosis, under in vitro conditions, confirm the results of our agitation experiments so far as the actions of heated and unheated sera are concerned.

Finally, it is interesting to note that McKendrick[9] in a theoretical and mathematical consideration of this subject concluded, in part: "the phenomenon of phagocytosis can be satisfactorily treated from the physical point of view as a random interfusion between two perfactly intermixed systems of particles, each of which is evenly distributed, in which ingestion takes place when individuals of opposite type have collided."

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[4] Jour. Am. Med. Assn., 1906, 46, p. 1407.
[5] Ibid., 1911, 57, p. 1579.
[6] Jour. Infect. Dis., 1906, 3, p. 683.
[7] Ibid., p. 689.
[8] Tr. Chicago Path. Soc., 1911, 8, p. 208.
[9] Science Progress in the Twentieth Century, London, 1914, 8, p. 497.

TECHNIC

For the most part, we have followed the regular procedure as described by Wright, with the exception that in most of the experiments the bacteria and leukocytes have been kept separated at various temperatures and then agitated together by running the mixture back and forth a given number of times in a standardized capillary pipette, as recommended by Wright, followed by the immediate preparation of smears. The smears were fixed with methyl alcohol and stained with aqueous toluidin blue (10 mg. to 100 c.c. distilled water). We were inclined to believe that the further agitation of the mixture during the process of making the blood films increases the degree of phagocytosis. We have not demonstrated this, but, on the other hand, we have some evidence that some of the best cells are lost during the process of smearing. In order to control the smeared preparations, we made wet-fixation films as follows:

Perfectly clean slides are placed in moist chambers (Petri dishes) and 5 or 6 drops of Ringer's solution placed on each slide which, if clean, will allow the fluid to spread immediately over the surface. These slides should be prepared shortly before they are to be used. Then, when the mixture has been made, a few drops of it are placed in the Ringer's and distributed over the surface of the slide and throughout the Ringer's by tipping the Petri dish two or three times. The leukocytes are then allowed to settle for five to ten minutes. The Petri dish is then placed on the flat surface of a block of ice and two or three minutes later three or four drops of 2 percent osmic acid are placed within the Petri dish beside the preparation. Fixation for ten to fifteen minutes is followed by thorough washing with absolute followed by 95 percent alcohol and water. The affinity of such (osmic) preparations for toluidin blue is markedly increased, and staining must be practically instantaneous in order to get preparations in which the bacteria can be counted with any degree of accuracy. If too deeply stained, they must be decolorized and restained. Such preparations have many advantages over the ordinary smears. The leukocytes often retain to a greater or less degree their normal spherical contour. One often encounters leukocytes which plainly show that the bacteria are adherent to the surface of the cell and have not yet been carried into the deeper layers of the cytoplasm. In properly stained and differentiated specimens, there is no difficulty in counting the bacteria even tho they occur at different depths in the cell. Here, too, one often encounters large and small mononuclears and eosinophils which have cocci adherent to them. In fact, with proper differentiation one can see a considerable number of bacteria sticking to the lymphocytes.

Most of our experiments were done with the staphylococcus pyogenes aureus, isolated about a month previously from a severe infection of the finger. The cultures were grown at the temperatures given for given lengths of time on + 1 agar slants aerobically. Suspensions were made in sodium chlorid solution or in Ringer's of the concentrations mentioned later and centrifuged to remove the coarser clumps. In Experiments 9, 10, and 20, an actively motile, non-sporogenic bacillus (B. fluorescens liquefaciens ?) isolated from potato was used.

The human leukocyte suspensions (including many erythrocytes) were prepared by collecting blood in 1.5 percent sodium citrate followed by washing three times with 0.85 percent. sodium chlorid solution (Experiments 1 and 2) or Ringer's 0.69 percent (Experiments 3-10) or Ringer's 0.86 percent (Experiments 11-22). The 0.69 Ringer's had the following composition: NaCl 0.65, KCl 0.02, $CaCl_2$ 0.025 percent; the 0.86 percent Ringer's, NaCl 0.8, KCl 0.02, $CaCl_2$ 0.02, $NaHCo_3$ 0.02 percent.

In order to make the various experiments as comparable as possible, the leukocytes and serum from one of us was used throughout the series, and this one made all the mixtures and agitations.

In determining the degree of phagocytosis, the average number of bacteria in fifty polymorphonuclear or transitional leukocytes was counted with a Zeiss 1.8 mm. apochromatic lens, No. 6 ocular, and a bright artificial light.

EXPERIMENTS

The following experiments show that on separate incubation followed by agitation marked "phagocytosis" is produced.

Experiment 5.—(1) Cocci in 0.69 Ringer's, (2) Same suspension of cocci with an equal volume of twenty-four-hour-old human serum, and (3) washed, human blood suspended in serum were incubated separately for fifteen minutes at 34 C. Nos. 1 + 3 and 2 + 3 were mixed in small tubes with capillary pipettes five times and slowly centrifuged once; they were mixed again five times, and smears and osmic preparations were made. Smears 1 + 3 showed 23 cocci per leukocyte; Smears 2 + 3 showed 41 cocci per leukocyte; the Osmic 1 + 3 showed 32 cocci per leukocyte; in the Osmic 2 + 3 the cocci were too crowded to count accurately. It will be noted that in Nos. 1 + 3 twice as many cocci were present as were in Nos. 2 + 3, and that here opsonization took place during the mixing and centrifuging. While counting the fifty cells in Smears 1 + 3 two eosinophils were encountered containing 8 and 12 cocci, respectively; likewise, in the osmic preparation eosinophils contained 6, 14, and 9 cocci; in Smears 2 + 3 eosinophils contained 32, 33, and 24 cocci.

Experiments 8, 10, and 11 show the effects of a minimum degree of agitation.

Experiment 8.—Washed (0.69 Ringer's) human corpuscles were suspended in an equal volume of twenty-four-hour-old serum. (2) A fairly dense emulsion of cocci in 0.69 Ringer's was diluted with an equal volume of the serum. Equal volumes of Mixtures 1 and 2 were then placed in a short 15 mm. test tube — the cocci being gently dropped into the blood and given a rotary shake two or three times and then incubated at 39 C. for 30 minutes. The precipitated sediment was then removed with a 3 mm. glass tubing in order to avoid the churning of the capillary pipettte, and smears and osmic preparations were made. The smears showed 1.2 cocci and the osmic 1.9 cocci per leukocyte.

Experiment 10.—The technic used in Experiment 8 was used with the substitution of the actively motile bacillus from potato. The mixture was incubated for fifteen minutes at 36 C. The smears showed 7 bacilli per leukocyte and the osmic 4.

Experiment 11.—The technic was that used in Experiment 8, excepting that 0.85 percent NaCl was used. The mixture was incubated for twenty minutes at 38 C. The smears showed 7 cocci per leukocyte and the osmic 4.

The following agitation experiments show the effect of dilution of the staphylococcus emulsion on the degree of adhesion when the leukocytes and bacteria are incubated separately and then mixed and smeared. The serum in these three experiments could act on the bacteria only during the few seconds taken in mixing — hence the comparatively low counts.

Experiment 13.—The coccus emulsion was diluted to one-half, one-tenth, and one-twentieth the density of a twenty-four-hour typhoid broth culture with 0.86 percent Ringer's. The washed corpuscles were suspended in an equal volume of eighteen-hour-old serum, incubated for fifteen minutes at 38 C., and again mixed before mixing with the bacteria. Equal volumes of the bacterial emulsions and leukocyte-serum mixture were mixed five times in capillary pipettes of approximately the same size and then smeared. The one-half dilution gave 3 cocci, the one-tenth 0.02, the one-twentieth 0.01 cocci per leukocyte. The osmic preparations gave 2, and 0.02. The last dilution was not counted.

Experiment 14.—The cocci had been kept seven days on + 1 agar at 21 C. The washed leukocytes in eigheen-hour-old serum were incubated at 37 C. for fifteen minutes. The coccus emulsion was diluted one-half, one-fourth, and one-eighth its original density and the mixing was performed with short, three-inch, capillary pipettes. Otherwise, the technic was similar to that used in Experiment 13. The one-half dilution gave 2.9 cocci, the one-fourth 1.1 cocci, and the one-eighth 1 coccus per leukocyte. The osmic preparations were not counted.

Experiment 15.—This duplicates Experiment 14, excepting that the forty-eight-hour-old serum was incubated with the leukocytes for fifteen minutes at 38 C. and that the coccus emulsion was finally diluted to one-half and one-fourth its original density. The mixture was churned ten times in six-inch capillary pipettes before smears were made. The one-half dilution gave 2.4 and the one-fourth 1.2 cocci per leukocyte.

Experiment 16 clearly shows that if more time is given for the action of the serum upon the bacteria more of the latter adhere to the leukocytes.

Experiment 16.—The serum was fresh — three hours on ice. The coccus suspension from Experiment 15 was used. Two volumes of this suspension were mixed with one volume of serum and kept at 21 C. for thirty minutes. A similar mixture of cocci and serum was kept at 21 C. for fifteen minutes and then at 38 C. for fifteen more minutes. Equal volumes of these two bacterial suspensions were then churned ten times in six-inch capillary pipettes with similar volumes of washed leukocytes which had been incubated in serum for fifteen minutes at 38 C. The 21 C. test gave in the smear 6.7 cocci and in the osmic 6.6 cocci per leukocyte. The 21 C. plus 38 C. test gave in the smears 6.4 cocci and in the osmic 6.4 cocci per leukocyte. The final dilutions of the coccus emulsions were here nearly one-half the original density and the figures show that with the greater time for opsonization more than twice as many cocci adhered.

During the counting of the 200 polymorphonuclears in this experiment, thirteen large mononuclears were seen with from 3-6 adherent cocci; twelve lymphocytes with from 1-9 adherent cocci and eight eosinophils with from 1-5 adherent cocci.

That the usual result produced by heating serum can be demonstrated by churning separately incubated mixtures of bacteria and serum and serum and leukocytes is shown by Experiment 18.

Experiment 18.—The cocci from a forty-eight-hour-old culture were suspended in 0.86 Ringer's. The serum was separated for forty-eight hours on ice, and half of it heated for fifteen minutes at 55 C. Equal volumes of the heated and unheated sera and of the coccus suspension were thoroughly mixed and kept at 21 C. for twenty-eight minutes. Likewise, equal volumes of thoroughly agitated washed leukocytes in heated and unheated sera were incubated at 38 C. for fifteen minutes. Four mixtures were then made by churning ten times in six-inch capillary pipettes and smears and osmic preparations made at once:

The cocci in unheated serum plus leukocytes in unheated serum gave in the smears, 9 cocci, and in the osmic, 8 cocci per leukocyte; the cocci in heated serum plus leukocytes in heated serum gave both in the smears 0.1 coccus per leukocyte and in the osmic 0.1; the cocci in the heated serum plus leukocytes in unheated serum gave in the smears 2 cocci and in the osmic 0.6 coccus per leukocyte; the cocci in unheated serum plus the leukocytes in heated serum gave in the smears 9.9 cocci and in the osmic 9.6 cocci per leukocyte.

The following experiments were performed to test the influence of temperature on the "opsonization" of bacteria by normal serum.

Experiment 19.—The cocci from a forty-eight-hour-old culture were suspended in 0.86 Ringer's and were chilled in the ice-box at 11 C. The serum which was three hours old was also chilled to 11 C. Three mixtures of equal volumes of bacteria and serum were then made; one was kept for forty-five minutes at 11 C.; another at 21 C. for thirty minutes and then for fifteen minutes at 11 C.; the last was kept at 36 C. for fifteen minutes and then at 11 C. for thirty minutes. Then, equal volumes of these suspensions were separately churned ten times in six-inch capillary pipettes with washed leukocytes which had been suspended in serum at 36 C. for fifteen minutes. Those kept at 11 C. gave in the smears 9.7 and in the osmic 8 cocci per leukocyte; those at 21 C. gave in the smears 9 and in the osmic 11 cocci per leukocyte; those at 36 C. gave in the smears 11 cocci and in the osmic 8.7 per leukocyte.

As it seemed rather surprising that "opsonization" should occur about as well at 11 C. as at 36 C., lower temperatures were tried as follows:

Experiment 20.—The potato bacillus in 0.86 Ringer's, and two-hour-old serum were kept in separate tubes in crushed ice at 1-2 C. In a few minutes, equal volumes of the two were mixed and one portion kept at 1-2 C. and the other at 21 C. for thirty minutes. Washed leukocytes suspended in serum were kept at 37 C. for fifteen minutes. Equal volumes of the two suspensions and of the leukocytes were then churned twelve times in six-inch capillary pipettes and smeared. That kept at 1-2 C. gave 2 bacilli, and that at 21 gave 9 bacilli per leukocyte.

Experiment 21.—The procedure of Experiment 20 was duplicated, excepting that a suspension of a twenty-four-hour-old culture of cocci was used and in this experiment the lower temperature was kept at 1 C. That kept at 1 C. gave 3 cocci while that kept at 21 C. gave 10.7 cocci per leukocyte. The osmic preparations were allowed to settle for too long a time (about twenty minutes) at 21 C. and when examined showed too much intracellular digestion for accurate counting. These preparations were similar to those encountered before in Experiment 6 and are particularly interesting in the fact that they demonstrate that the cocci are actually rolled into the cytoplasm of the cell and digested there. In many instances, cells were found with the intracellular cocci appearing as mere shadows whereas the cocci, still adherent to the surface of the leukocyte, appeared normal and stained intensely.

Only a single experiment was done on the possible influence of temperature on the leukocytes, and this experiment is not satisfactory so far as the lowest and highest temperatures are concerned, as precautions were not taken to collect and wash the leukocytes at a temperature other than that of the room, which was that of a warm day, 26 C.

Experiment 22.—The technic was that used in Experiment 20, excepting that the cocci plus serum were kept at 26 C. for thirty minutes and the three lots of the thoroughly mixed leukocytes were kept at 1, 26, and 38 C. for fifteen minutes. In the 1 C. experiment, the smears gave 9 and the osmic 13 cocci per leukocyte; in the 26 C. test the smears gave 10.8 and the osmic 13.9 cocci per leukocyte; in the 38 C. test the smears gave 11 and the osmic 14.7 cocci per leukocyte.

However, this experiment shows that it is hardly necessary to incubate the leukocyte serum mixture before agitation — as done by us in this series of experiments.

In the following experiment, frog's leukocytes were used.

Experiment 9.—Frog's leukocytes were collected by injecting a suspension of aleuronat into the peritoneal cavity, collecting some of the exudate in sodium citrate, and washing three times with 0.69 Ringer's. This suspension, containing many clumped leukocytes, was then mixed with a fairly dense suspension of the actively motile potato bacillus (in 0.69 Ringer's) and allowed to stand at 21 C. for thirty minutes. Then, some of this mixture was agitated thoroughly in a capillary pipette and smears and osmic preparations were made. The bacteria in 100 leukocytes in each preparation were then counted. The smears gave 2.8 bacilli and the osmic 3 bacilli per leukocyte. In the osmic preparations it was noted that the leukocytes which were massed together in clumps often showed a particularly high degree of adhesions, for example, the counts on the individual cells of five such clumps are as follows: (a) 28, 43, 25, 20; (b)

5, 23, three leukocytes too dark to count; (c) 0, 2, 28, two leukocytes too dark to count; (d) 0, 0, 0, 0; (e) 0, 13, 0, 0, 0, 26. In a control microscopic preparation of this mixture, kept at 21 C., we were unable tō detect any signs of phagocytosis altho some cells were watched for about an hour. Frequently, the actively motile bacilli bumped into the leukocytes but only occasionally stuck for a short time and then broke loose and escaped. This experiment shows clearly that some leukocytes, at least, are capable of having some bacteria adhere to them without the interaction of serum, provided that the bacteria are brought into contact with their surfaces with sufficient force.

DIRECT OBSERVATIONS ON THE MECHANISM OF "PHAGOCYTOSIS"

We have placed mixtures of leukocytes, serum and cocci on a modified Barber moist chamber[10] at 23 C., and, by means of fine glass needle (1-2 μ) manipulated in the Barber pipette holder, have pushed cocci against leukocytes, and then, by traction on the cell, have seen that they adhere when unheated serum is used, and that they do not adhere when serum heated to 55 C. for fifteen minutes is used. Again, leukocytes have been dragged through such emulsions and in the one case they pick up many cocci while in the other they do not. Cocci picked up in this way seem to be rolled into the interior, and in the case of one cell we watched the digestive vacuoles form about the enclosed cocci which in less than an hour had dissolved and disappeared.

In the preparation containing washed leukocytes, heated serum, and cocci, we found one large mononuclear with eight or ten adherent cocci which could not be dislodged by dragging the leukocyte about. Other leukocytes which were dragged about in the same preparation did not have cocci adhere to them. This further supports the idea that some leukocytes are sticky enough for bacteria to adhere to them without the interaction of serum.

SUMMARY AND CONCLUSIONS

Following observations on the adhesive character of the surface of certain amebae and certain vertebrate leukocytes by Kite, experiments were undertaken which show that when separately incubated mixtures of leukocytes and serum and serum and bacteria are agitated together many of the bacteria stick to the leukocytes and are rolled into their substance. That the bacteria are actually within the cells is judged by observations on their digestion as shown both in fresh and in stained preparations. By this method, several of the classical experiments on phagocytosis have been repeated. Experiments with a minimum amount of agitation show that here "phagocytosis" is reduced.

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[10] See Kite: Amer. Jour. Physiol., 1913, 32, p. 146.

We offer the suggestion that foreign particles, as carbon, are taken up by leukocytes because the latter have sticky surfaces; that bodies similar to many bacteria stick to leukocytes best in the presence of unheated serum because they adsorb something from the serum which makes them more sticky or are in some way rendered more sticky, and hence the chances of their adhering to the surfaces of leukocytes are increased. There is evidence that, even in the absence of serum, certain leukocytes are sticky enough to allow some bacteria to adhere. Preliminary experiments on the influence of temperature on the power of serum to make staphylococci sticky seem to indicate that this may occur as well at 11 C. as at 37 C., but that the action is diminished considerably at 1 C.